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PRINCIPAL INVESTIGATOR: Sandra W. McLeskey, Ph.D.

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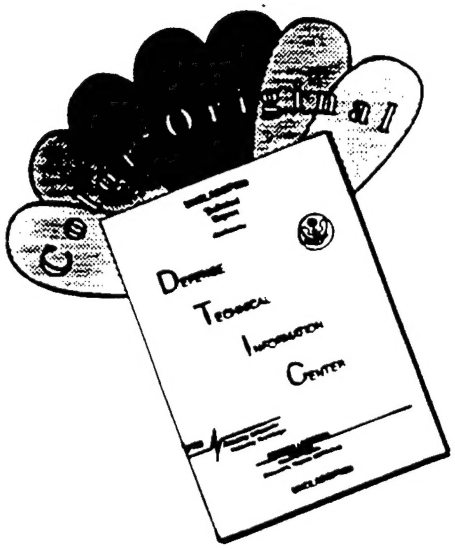

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13. ABSTRACT *(Maximum 200*

Metastatic estrogen receptor positive breast carcinoma may be treatable with tamoxifen, an antiestrogen, but the tumor may subsequently become refractory to treatment, growing in the absence of estrogenic stimulation. We have developed a model of breast cancer progression by transfecting MCF-7 breast carcinoma cells, which are estrogen-dependent for growth in ovariectomized nude mice, with cDNAs for FGF-1 or FGF-4. The transfected cell lines are able to form tumors in ovariectomized nude mice. Since FGFs are angiogenic factors, this project investigates the importance of angiogenesis in the phenotypic transition of the transfected cells by looking for differences in the angiogenesis in tumors produced by the parental MCF-7 or the FGF-transfected cells. Our model is validated by a positive correlation of tumor microvessel density and tumor size. We have defined temporal and spatial events in the process of tumor-induced angiogenesis by identifing sprouting or proliferating endothelial cells. We have identified patterns of angiogenesis which are associated with regressing parental cell tumors and growing FGF-transfected cell tumors. We have begun to isolate endothelial cell populations from parental or FGF-transfected cell tumors to study FGF receptor gene expression. Future work will study gene expression in endothelial cells with a combination of *in vivo* and *in vitro* methods.

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Sandra W. McLeskey 8/12/96

PI - Signature Date

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INTRODUCTION

When initially diagnosed, human breast cancer may be estrogen receptor positive and amenable to hormonal therapy with the antiestrogen, tamoxifen. However, eventually such tumors may become refractory to tamoxifen treatment, progressing to an invasive, metastatic, phenotype that is essentially untreatable (1). We have developed a model tumor system that mimics some aspects of breast cancer progression to a tamoxifen-resistant, more metastatic state, by transfection of fibroblast growth factor 4 (FGF-4) into estrogen-dependent MCF-7 cells. However, the dramatic change to a progressively growing, antiestrogen-resistant and metastatic phenotype produced by this transfection *in vivo* is not paralleled by a similar change in the *in vitro* phenotype of the transfected cells (2-5). Although growth requirements *in vitro* and *in vivo* may be somewhat different, this discrepancy between *in vitro* and *in vivo* behavior of these cells points to stromal factors as mediators of the tumorigenic, antiestrogen-resistant, metastatic phenotype in this system. Since FGFs are angiogenic growth factors, increased angiogenesis produced by the transfected cells may be responsible for this change in tumor phenotype. This project attempts to validate this system as a model of tumor angiogenesis and seeks to identify endothelial cell responses to FGF-4 which are important in mediating the tumorigenic, metastatic phenotype of these cells. This report covers work done in the second year of the project.

During the first year, techniques were developed which enabled double staining of BrdU (bromodeoxyuridine) labeled nuclei to identify proliferating cells, and PECAM (platelet-endothelial cell adhesion molecule, CD31, an endothelial-specific marker) to identify endothelial cells, in the same pathological section. This technique has allowed us, during the second year, to draw conclusions regarding the proliferative state of tumor or endothelial cells, revealed by BrdU labeling, relative to blood vessel morphology and distribution, as revealed by PECAM staining as delineated in Aim 2. These results are described below.

Also during the first year, techniques were developed for isolation of tumor-derived endothelial cells from experimental tumors. These techniques have been further refined during the second year, and we are almost ready to perform our subtractive cloning utilizing ribonucleic acid (RNA) obtained from the isolated cells (Aim 3). The results concerning these techniques are described below.

Additionally during the second year, we have expanded our studies to include FGF-1 transfected MCF-7 cells. These cells have an *in vivo* phenotype which is similar, but not identical to the FGF-4 transfectants (5). They are now being included in the project since FGF-1 has been found to be expressed in human breast cancer, but FGF-4 has not (6). Thus, we feel that inclusion of these transfectants lends validity to our findings.

BODY

Aim 1 of the project, which was to correlate tumor size and metastasis with microvessel

density, was essentially achieved in the first year utilizing archival tumor specimens from experiments investigating the effect of antiangiogenic drugs on tumors produced by the FGF-transfected MCF-7 cells (7). Although we plan to repeat experiments correlating tumor size and metastasis with microvessel density in the near future, we wish to use image analysis to quantitate the metastasis and the maximal microvessel density. We are in the process of developing image analysis techniques to do this in connection with another project, and will defer completion of this aim until these techniques are in place.

In aim 2, we hypothesized that there would be a temporal and spatial difference between neovascularization stimulated by FGF-4 transfected or parental MCF-7 cells in tumor nodules produced early after tumor cell injection into ovariectomized nude mice. We felt that temporal or spatial differences in endothelial cell proliferation or tumor cell proliferation would prove to be important in determining the success of the tumor in an ovariectomized animal. Surprisingly, although we found differences in the number of neovessels in tumors produced by FGF-4 transfectants vs those produced by parental cells, the most striking differences concerned the distribution and morphology of neovessels in the two tumor types. Immediately following tumor cell injection, tumor nodules produced by FGF-4 transfected, FGF-1 transfected or parental cells in the mammary fat pads of ovariectomized nude mice contain neovessels interspersed with tumor cells. Although tumor nodules produced by the FGF transfectants contain more neovessels than those produced by parental cells, parental cell nodules contain many neovessels. Beginning about 10 days following tumor cell injection, neovessels in tumor nodules produced by parental cells began to assume a dilated, irregularly-shaped morphology surrounding the nodule, while FGF-transfected tumor nodules contained small-caliber neovessels intimately associated with and interspersed among the tumor cells. By 15 days following tumor cell injection, parental cell nodules were regressing, as evidenced by low levels of BrdU incorporation, and contained many dilated, irregularly shaped vessels surrounding the tumor with intervening stroma between the vessels and nodules of tumor cells. FGF-transfected tumors, which were actively growing as indicated by a high level of BrdU labeling, continued to exhibit small caliber neovessels intimately associated with tumor cells. Thirty-five days after tumor cell injection, parental cell tumor nodules were undetectable by palpation but could be revealed by X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) staining since the cells are transfected with bacterial *lacZ*. In contrast, tumors produced by FGF-4 transfected cells continue to grow, exhibit a high level of BrdU labeling, and have small caliber neovessels intimately associated with tumor cells. Figure 1 depicts tumor nodules produced by parental cells and actively growing tumors produced by FGF-1 and FGF-4 transfected cells at different time points illustrating the different morphologies and distribution of neovessels. We are currently in the process of subjecting pathological sections of tumors produced by parental MCF-7 or FGF-transfected cells at critical time points to image analysis procedures. We wish to quantify the number of dilated, irregularly-shaped neovessels around the tumor vs the number of small caliber neovessels intimately associated with the tumor. The number of each type of vessel will be correlated with the BrdU labeling in each type of tumor.

Figure 1

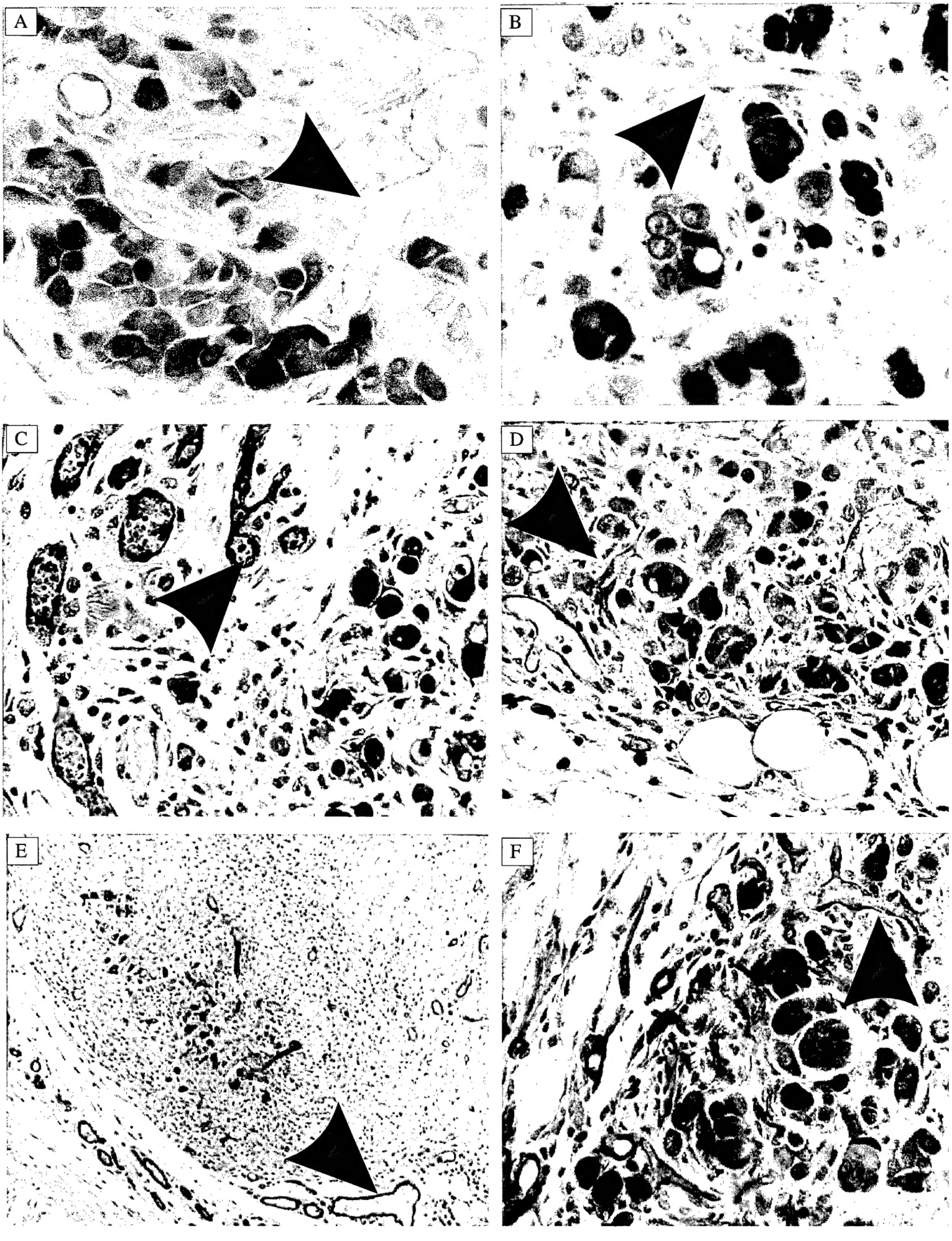

FIGURE 1: Patterns of neovascularization after injection of parental MCF-7 and FGF-transfected MCF-7 cells into the mammary fat pads of ovariectomized nude mice. Tumors or tumor nodules produced by injection of parental MCF-7 (A, C, and E) or FGF-4 transfected (MKL-F) (B, D, and F) cells were harvested at days 6 (A and B), 10 (C and D), and 35 (E and F), stained with X-gal (blue) to reveal tumor margins, embedded in paraffin, sectioned, and subjected to double immunohistochemistry for BrdU (red) and murine PECAM-1 (brown). (Since the tumors were stained with X-gal when whole, only surface tumor cells stain blue.) Arrowheads point to blood vessels typical of neovessels in regressing tumors (C and E) or growing tumors (A, B, D, and F).

As mentioned in the progress report last year, we have been somewhat thwarted in our efforts to demonstrate the presence of ETS-1 (8), a transcription factor reported to be expressed in developing vessels, in pathological sections of tumors produced by FGF-transfected or parental MCF-7 cells. Immunohistochemistry utilizing a polyclonal rabbit antibody raised to human ETS-1 was not successful because of nonspecific staining in many cells known not to express ETS-1. Consequently, we have turned to *in situ* hybridization to reveal expression of this factor. We are endeavoring to develop a sensitive nonradioactive method of *in situ* hybridization in our laboratory. This method utilizes a digoxigenin-labeled riboprobe with subsequent immunohistochemistry using an anti-digoxigenin sheep antibody followed by a biotinylated anti-sheep secondary antibody and an avidin-biotin complex (ABC) coupled to horseradish peroxidase. The horseradish peroxidase is used to precipitate diaminobenzidine into the tissue. The sensitivity afforded by this method should be able to reveal the presence of low copy number transcripts which would be characteristic of transcripts of transcription factors or other regulatory proteins. We feel it is imperative to develop this nonradioactive method for *in situ* hybridization for two reasons. First, it is considerably less expensive than radioactive methods. (Funds for supplies for the research carried out in this program are provided by the PI's NIH FIRST award.) Second, it eliminates the need to dispose of radioactive waste, which saves institutional money as well as other resources. Development of this nonradioactive method has been supported by a $750 grant-in-aid from the Dean of Research of Georgetown University. So far, we have been successful in performing *in situ* hybridization with a digoxigenin-labeled riboprobe for human glyceraldehyde 3-phosphate dehydrogenase, a very abundant transcript which is present in the tumor cells but not the stromal cells in our pathological sections. To do this, we utilized alkaline phosphatase labeled F_{ab} fragments directed against digoxigenin. Our plans are to add the amplifying power of a biotinylated secondary antibody followed by ABC to increase the sensitivity of our assay before switching to an *ets-1* riboprobe.

A major effort in this year has been the isolation of tumor-derived endothelial cells. We have succeeded in isolating these cells from tumors produced by FGF-4 transfected cells by performing fluorescence-activated cell sorting (FACS) using two rat monoclonal antibodies for murine PECAM, both coupled to fluorescein isothiocyanate (FITC). By using two different monoclonal antibodies, which presumably bind to different epitopes of the PECAM molecule, we are able to boost the signal we obtain in FACS so that the selected cells have two logs brighter fluorescence than the majority of cells in the tumor. Figure 2 shows the FACS histograms of the tumor cells without the PECAM

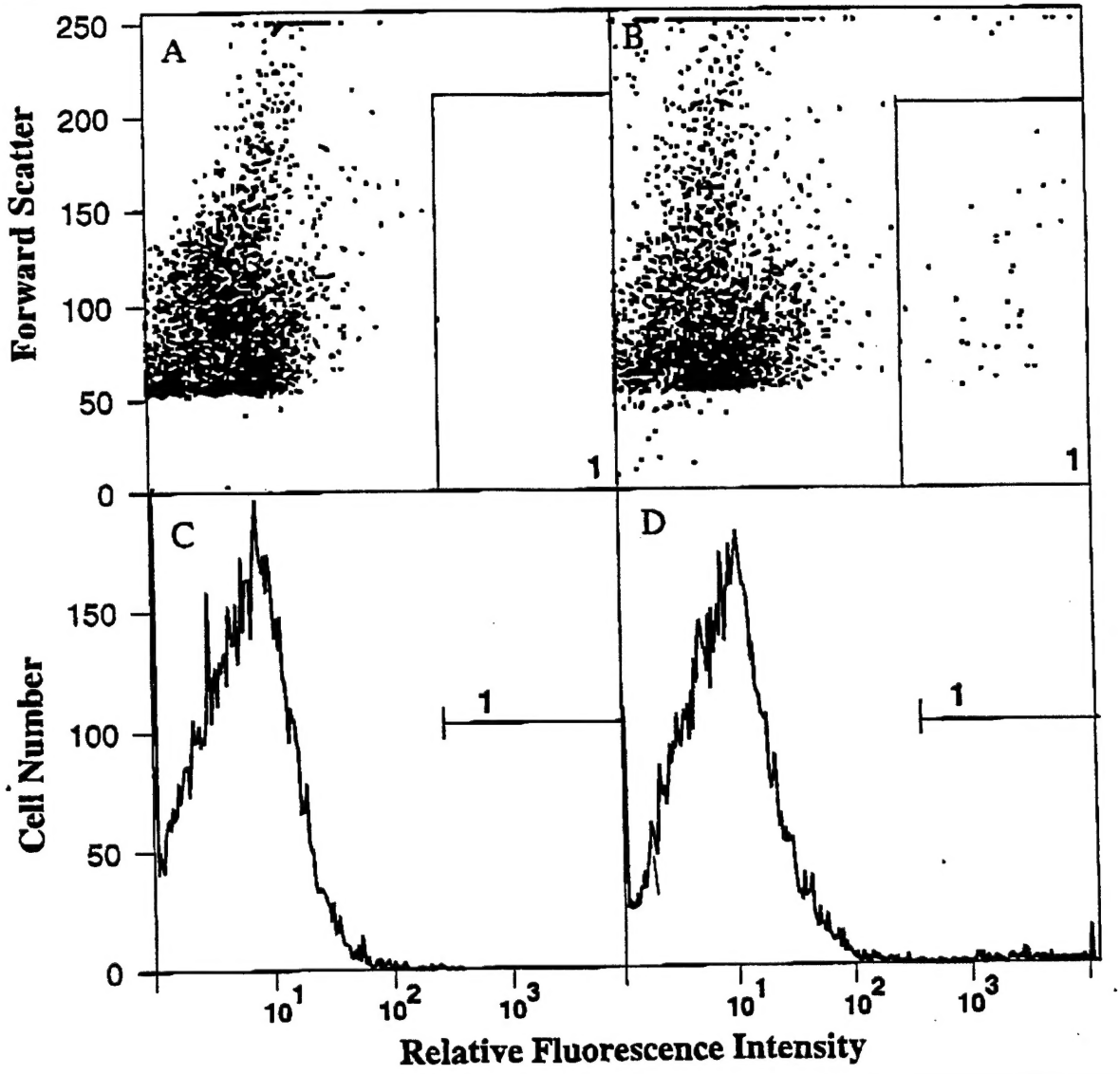

Figure 2. FACS analysis of cells obtained from a digested tumor. A and B: Plots of relative fluorescence intensity vs forward scatter, an indicator of cell size. Gates for sorting were selected to discard large cells or clumps of cells. C and D: Plots of relative fluorescence vs cell number. Gates were selected to pick out the cells which were two logs brighter than the peak fluorescence of the negative control. A and C: Cells sorted without addition of FITC-coupled antibodies to PECAM. B and D: Cells sorted after incubation with two rat monoclonal antibodies to murine PECAM. Lines denoted "1" indicate the selection gates. Approximately 1.4% of sorted cells were selected.

antibodies (the negative control) and the histograms obtained when the antibodies were used. Since

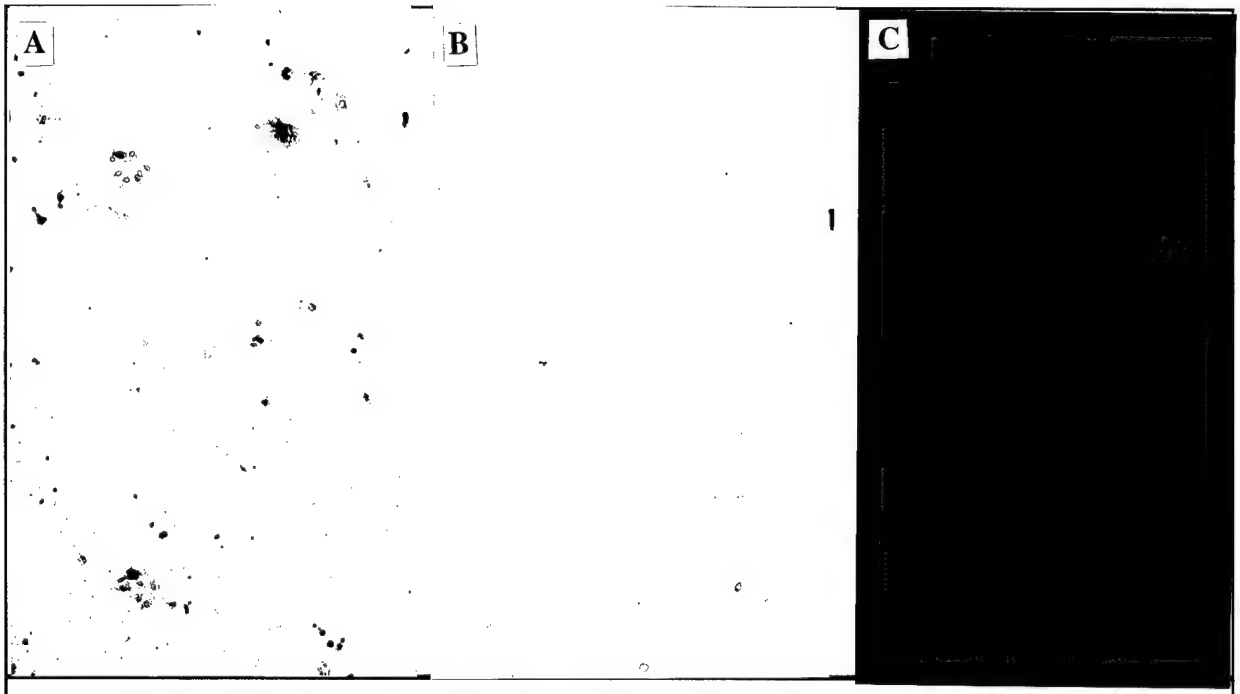

Figure 3. The selected cells have endothelial characteristics. Immunohistochemistry and LDL uptake were performed on a few cells which were plated following FACS. A and B: Cells stained with a rat monoclonal antibody for PECAM. A: The selected cells from FACS. B: The rejected cells from FACS. C: Fluorescent LDL (acetylated LDL coupled to 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylcarbocyanine perchlorate, Molecular Probes) uptake by the selected cells.

this experiment was done, we have obtained a mouse hemangioma cell line, EOMA (9) which expresses PECAM and can be used as a positive control for both FACS and immunohistochemistry. The isolated tumor-derived endothelial cells, when plated, have a high incidence of expression of PECAM on immunohistochemistry and most of them are capable of LDL (low density lipoprotein) uptake (Figure 3). These two characteristics imply that the selected cells are of endothelial origin. In a preliminary, proof-of-principle experiment, RNA was purified from 100,000 isolated cells. Two micrograms of RNA were obtained, of which 0.5 μg RNA was subjected to denaturing electrophoresis and demonstrated intact ribosomal bands (Figure 4). This RNA will be used in RT-PCR (reverse transcription, polymerase chain reaction) to demonstrate the endothelial nature of the isolated cells.

Because success in subtractive cloning depends upon having RNA populations which are the same in every respect except the difference of interest, it is very important that the RNA isolated from the tumor-derived endothelial cells from FGF-transfected tumors or parental cell tumors contain the same amount and kind of contaminating cells not of endothelial origin. Therefore, we

have devised a scheme by which we can estimate the number of contaminating cells in the RNA we obtain from endothelial cells isolated from tumor produced by parental MCF-7, FGF-1 or FGF-4 transfected cells, or from mammary fat pads. To show that the RNA comes from endothelial cells, we will perform RT-PCR for murine PECAM. This transcript should be present at high levels in endothelial cells and absent in tumor cells or fibroblasts. (Although PECAM has been shown to be expressed in some tumor cells by immunohistochemistry or RT-PCR (10,11), it is not expressed in our experimental tumor cells.) Contaminating immune cells, such as macrophages, should express PECAM at much lower levels. As a positive control for this transcript, we have RNA isolated from EOMA cells mentioned above. In addition, as negative controls, we have RNA isolated from NIH 3T3 mouse fibroblasts and from the MKL-F clonal cell line of FGF-4 transfected MCF-7 cells. Figure 5 shows RT-PCR from these cell lines demonstrating the presence of PECAM transcripts in RNA isolated from tumor-derived endothelial cells selected from a tumor produced by the MKL-F clonal line of FGF-4 transfected MCF-7 cells.

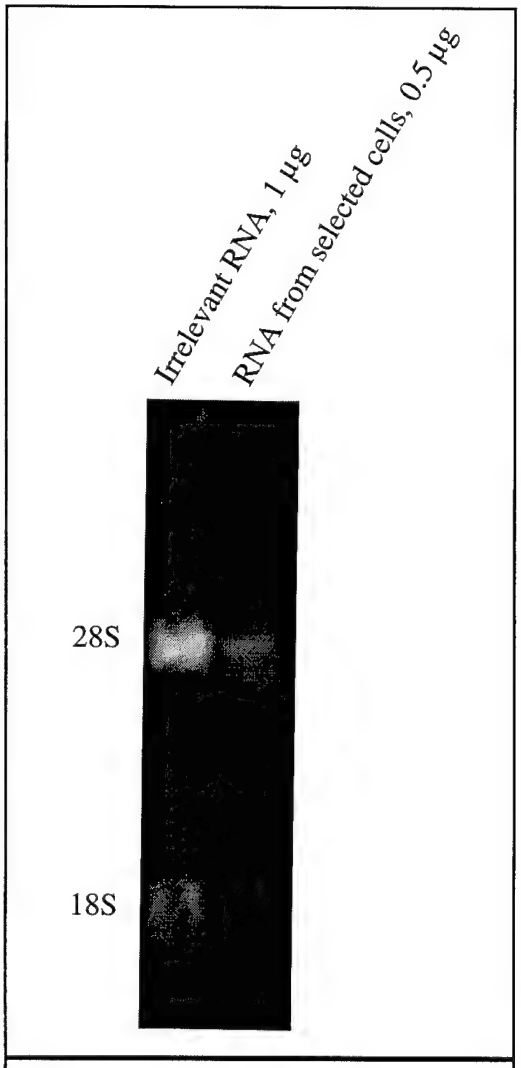

Figure 4. RNA extracted from the selected cells exhibited intact ribosomal bands. Total RNA was subjected to denaturing agarose gel electrophoresis. Lane 2 contains 0.5 µg of the RNA purified from the selected cells. Lane 1 contains 1 µg of an irrelevant RNA.

To show the absence of tumor cell RNA in the RNA purified from the tumor-derived endothelial cells, we are performing RT-PCR with primers specific for human KGFR (keratinocyte growth factor receptor). This transcript is a splice variant of mRNA for FGF receptor 2 (12) and is expressed in epithelial cells, including MCF-7 cells and the FGF transfected MCF-7 cells (6). It should not be expressed in endothelial cells because it is an epithelial cell receptor and because it is a human transcript. RNA isolated from MKL-F cells will serve as a positive control for this experiment and NIH-3T3 and EOMA cells will be negative controls.

We have had difficulty identifying a protein which is expressed by fibroblasts but not by endothelial cells. Since endothelial cells have a similar mesodermal origin to fibroblasts, they express many of the same proteins. We think we will be able to demonstrate the absence of

contaminating fibroblast RNA in the RNA purified from the tumor-derived endothelial cells by performing RT-PCR with primers specific for FSP1, a protein which does not seem to be expressed by mouse endothelial cells, at least by immunohistochemistry (13).

When we are able to estimate the degree of contaminating cells in the RNA obtained from tumor-derived endothelial cells, we will proceed with our subtractive cloning. We are fortunate to have a collaboration with Human Genome Sciences, Gaithersburg, MD, where the subtractive cloning will be done. The cloning will be done by the SAGE (serial analysis of gene expression) method (14). Four thousand sequence tags will be identified and a profile of RNA expression determined for endothelial cells isolated from tumors produced by the MKL-F clonal FGF-4 transfected cells, parental MCF-7 cells, and from endothelial cells isolated from mammary fat pads of uninjected mice. We expect to deliver RNA to Human Genome Sciences for

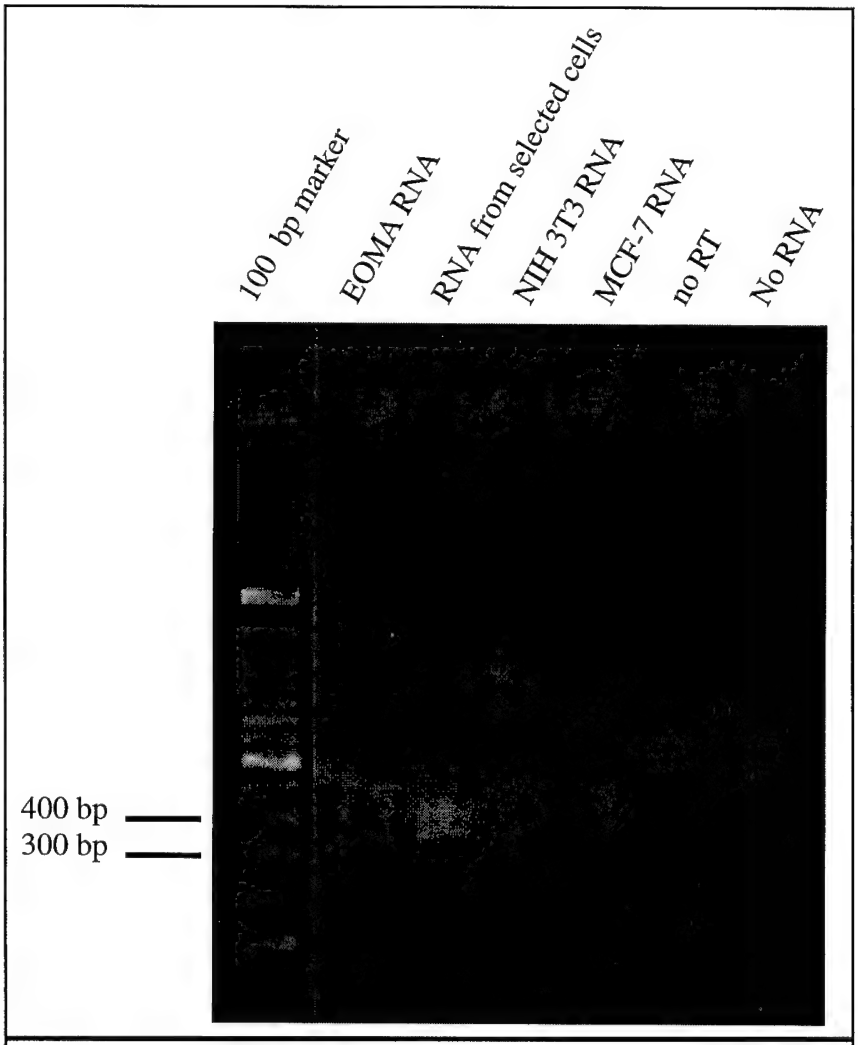

Figure 5. RNA from selected cells contains transcripts for murine PECAM. Total RNA (0.05 μg) was subjected to RT-PCR using primers specific to murine PECAM which are predicted to generate a 417 bp fragment of the cDNA. The RT-PCR conditions were adjusted so that amplification was still proceeding logarithmically at the end of the reaction. When RNA purified from a mouse hemangioma cell line, EOMA, was used as template (Lane 2), a doublet is generated with the 417 bp predicted fragment as the slower mobility member. A doublet of similar mobility and intensity is generated when RNA from the selected cells is used (Lane 3). No amplified product is seen when NIH 3T3 murine fibroblast RNA (Lane 4) or human MCF-7 breast carcinoma cell RNA (Lane 5) are used as templates. Lane 6 contains a similar RT-PCR reaction utilizing EOMA cell RNA but with the reverse transcriptase enzyme omitted. Lane 7 contains a similar RT-PCR reaction without template RNA.

analysis in late 1996.

CONCLUSION

We have made significant progress in accomplishing the aims of the project. They all ought to be accomplished easily by the end of the project period.

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APPENDIX

LIST OF ABBREVIATIONS

ABC	Avidin-biotin complex.
a.k.a.	Also known as.
BrdU	Bromodeoxyuridine.
CD31	The human homolog of PECAM (below). CD stands for clusters of differentiation.
EOMA	A mouse hemangioma cell line.
ETS-1	A transcription factor.
F_{ab}	Fragment of an antibody which includes the epitope binding region but not the constant (F_c) region.
FACS	Fluorescence-activated cell sorting.
FGF	Fibroblast growth factor
FGF-1	Fibroblast growth factor 1, a.k.a. acidic FGF.
FGF-1,clone 18	A clonal cell line of ML-20 cells (below) transfected with FGF-1.
FGF-4	Fibroblast growth factor 4, a.k.a. Kaposi FGF/HST-1
FGFR	FGF receptor.
FIRST award	First independent research support and transition award
FITC	Fluorescein isothiocyanate
FSP1	Fibroblast specific protein=1 (a.k.a. *pEL-98*, , and *mts1*).
KGFR	Keratinocyte growth factor receptor.
MCF-7	An estrogen receptor positive breast carcinoma cell line developed at the Michigan Cancer Foundation
MKL-F	A clonal cell line of MCF-7 cells cotransfected with FGF-4 and *lacZ.*
MKL-4	A clonal cell line of MCF-7 cells cotransfected with FGF-4 and *lacZ.*
ML-20	A clonal cell line of MCF-7 cells transfected with *lacZ.*
NIH	National Institutes of Health
NIH 3T3	A cell line of immortalized mouse fibroblasts.
PECAM	Platelet-endothelial cell adhesion molecule
PI	Principle investigator
RT	Reverse transcriptase
RT-PCR	A reverse transcription followed by the polymerase chain reaction.
SAGE	Serial analysis of gene expression
X-gal	5-bromo-4-chloro-3-indoyl-β-galactopyranoside, a chromogenic substrate for β-galactosidase

Meeting abstracts since the last report:

Bryan, J.A., Tobias, C.A., and **McLeskey, S.W.** A model for early events in tumor-induced angiogenesis. 85th Annual Meeting of the United States and Canadian Academy of Pathology, Washington, DC, March 23-29, 1996.

Tobias, C., Bryan,J.A., Filie, A., and **McLeskey, S.W.** Elucidation of early events in tumor-induced angiogenesis. 87th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 20-24, 1996.

Publications since the last report:

McLeskey, S.W., Zhang, L., Kharbanda, S., Liu, Y., Trock, B.J., Gottardis, M.M., Lippman, M.E., and Kern, F.G. Effects of AGM-1470 and pentosan polysulfate on tumorigenicity and metastasis of FGF-transfected MCF-7 cells. 1996, Br J. Cancer, **73**, 1053-1062.

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McLeskey, S.W., Zhang, L., Trock, B. J., Kharbanda, S., Tobias, C. A., Dickson, R. B. and Kern, F.G. Extraovarian estrogen production is not responsible for the estrogen-independent growth of FGF-transfected MCF-7 cells. Manuscript in preparation.

Tobias, C. A., Bryan, J. A., Vezza, P. R., Filie, A. F., and **McLeskey, S. W.** Elucidation of early events in tumor-induced angiogenesis. Manuscript in preparation.

Personnel receiving pay from this negotiated effort:

Sandra W. McLeskey, PhD